Development of an *In Vitro* Model to Study Oxidative DNA Damage in Human Coronary Artery Endothelial Cells

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Summary
The endothelial cell layer is a multifunctional barrier between the blood stream and the vascular wall. Reactive oxygen species can damage endothelial cells and may so potentiate atherosclerotic lesion formation. Therefore, we established an in vitro model for the qualitative and quantitative investigation of oxidative DNA damage and repair in human coronary arterial endothelial cells (CAEC). Oxidative DNA damage was induced by standardised treatment with 50-400 µM hydrogen peroxide (H_2O_2). The amount of DNA damage was quantified by determination of DNA single strand breaks (SSB) and alkali-labile sites in individual cells, using the alkaline single-cell gel electrophoresis assay – "comet assay". Significant DNA damage could be induced reproducibly in CAEC cells after exposure to 50 µM H_2O_2 for 10 min. Additional treatment with catalase prevented DNA damage by H_2O_2. The time needed for DNA repair depended on the initial grade of damage. After 30 min post-incubation at 37°C, DNA damage was completely repaired in cells treated with 50 µM H_2O_2. Cell samples initially damaged with H_2O_2 concentrations between 100 µM and 400 µM were repaired after 60 min. This endothelial cell culture model allows experiments on oxidative DNA damage, alteration, and repair directly on the relevant target cells. Animals are used neither as direct objects of such experiments nor as cell or tissue donors.

Zusammenfassung: Etablierung eines *in vitro* Modells zur Untersuchung oxidativer DNA Schäden in menschlichen koronaren Endothelzellen
Die Zellen des Endothels bilden eine multifunktionelle Barriere zwischen Blutstrom und Blutgefäßwand. Freie Radikale können diese Zellen schädigen und dadurch auch die Entwicklung von atherosklerotischen Veränderungen fördern. Deshalb haben wir versucht, ein in vitro Modell für die qualitative und quantitative Untersuchung von oxidativen DNA-Schädigungen in menschlichen koronaren Endothelzellen (CAEC) zu etablieren. Die oxidative DNA-Schädigung wurde durch standardisierte Behandlung mit 50-400 µM Wasserstoffperoxid (H_2O_2) erzeugt. Der Grad der DNA-Schädigung in einzelnen Zellen wurde über DNA-Einzelstrangbrüche und alkali-sensitive Stellen quantifiziert. Dafür wurde die alkalische Einzelzell-Gelelektrophorese-Technik (comet assay) eingesetzt. Durch eine Behandlung der Endothelzellen mit 50 µM H_2O_2 über 10 min wurde reproduzierbar eine signifikante DNA-Schädigung erreicht. Eine gleichzeitige Behandlung der Zellen mit Katalase verhinderte die DNA-Schädigung. Die Zeit, die zur vollständigen Reparatur der DNA gebraucht wurde, entsprach dem Grad der vorherigen Schädigung. Nach 30-minütiger Inkubation der Zellen bei 37°C war die DNA-Schädigung durch 50 µM H_2O_2 vollständig repariert. Zellen, die mit 100-400 µM H_2O_2 behandelt worden waren, benötigten für die Reparatur bis zu 60 min. Mit diesem Endothelzell-Modell können systematische Untersuchungen über oxidative DNA-Schädigungen, ihre Beeinflussung und Reparation direkt an den menschlichen Zielzellen durchgeführt werden. Tiere werden dadurch weder direkt noch als Zell- oder Organspender benötigt.

Keywords: 3R, coronary vascular endothelial cells, atherosclerosis, oxidative DNA damage, comet assay

1 Introduction

The endothelial cell layer is a multifunctional barrier between the blood stream and the vascular wall. The role of the endothelium has been investigated with regard to vascular lipid accumulation and development of atherosclerosis (Ludden et al., 1942). The endothelium plays a role in vascular tone regulation, (anti-) coagulation, and exchange of molecules between the blood stream and deeper cell layers of the vascular wall and different tissues. Recent investigations focus on molecular and receptor mediated control mechanisms (Finking et al., 2001a; Fuster et al., 1992a; Fuster et al., 1992b; Ross, 1993; Ross, 1999). Both, morphological and functional integrity of endothelial cells are essential for normal vascular function (Finking et al., 2001b; Krasinski et al., 1997). In addition to mechanical injury, a number of chemical agents are supposed to be responsible for disturbing the endothelium, especially components of cigarette smoke. Reactive

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oxygen species (ROS) are frequently found as products of metabolic or inflammatory processes. They play an important role as second messengers, mediating short-term signalling events (Sundaresan et al., 1995). On the other hand, reactive oxygen species induce cellular damage and may potentiate atherosclerotic lesion formation (Andreassi, 2003; Andreassi et al., 2000; Ballinger et al., 2000). However, little is known about DNA injury and repair kinetics in endothelial cells. On the background of our previous experiments with human vascular smooth muscle cells (Kreja and Finking, 2002) we have now established an *in vitro* model for the qualitative and quantitative investigation of oxidative DNA damage and repair in human coronary endothelial cells.

Oxidative DNA damage was induced by standardised hydrogen peroxide treatment. H_2O_2 has been established as a potent and direct agent of oxidant-induced DNA-strand breaks (Baker and He, 1991). The amount of DNA damage was quantified by determination of DNA single strand breaks (SSB) and alkali-labile sites in individual cells, using the alkaline single-cell gel electrophoresis assay – comet assay (Collins, 2002; Rojas et al., 1999; Singh et al., 1988; Tice et al., 2000). Several investigators have modified the assay to enable the detection of specific classes of DNA damage (review Tice et al., 2000; Collins et al., 1993; Collins et al., 1997; Merk and Speit, 1999).

2 Methods

2.1 Cell culture

Normal human coronary artery endothelial cells from one donor (43 years old, female) were purchased from Clonetics®/BioWhittaker Europe at third passage together with the endothelial growth medium including growth factors (EGM-2-MV Bullet Kit™) and a ready-made trypsin solution (ReagentPack™). For the stock culture the cells were cryopreserved after cultivation in 25 cm^2 bottles (Nunc, D-Wiesbaden) at 37°C in a humidified atmosphere with 5% CO_2 following the supplier's directions. All experiments were performed on cells at passage 5-7 which were grown to 80-90% confluence.

2.2 Treatment with hydrogen peroxide (H_2O_2)

Coronary artery endothelial cells (CAEC) were seeded in triplicate into 24-well plates (10.000-15.000 cells/well in 2 ml growth medium). Exponentially growing cells were washed with 2 ml HEPES buffered saline (UltraSaline A, BioWhittaker, Europe) and exposed for 10 min at 4°C, 20°C or 37°C to various concentration of H_2O_2 applied in 0.4 ml UltraSaline A. In some experiments the ROS scavenger catalase (285 U/ml; Sigma C-30) was added to the culture. After treatment the cells were washed twice, trypsinised, centrifuged (300g, 7 min), resuspended in medium and immediately used in the comet assay.

For the investigation of the damage repair kinetics, the cells were exposed to H_2O_2 for 10 min at 20°C, then washed twice, trypsinised, embedded in agarose on a microscope slide, and lysed immediately (0 min) or allowed to incubate in 2 ml of fresh culture medium for a period of 15-240 min at 37°C and 5% CO_2 before being embedded in agarose and lysed.

2.3 Alkaline single-cell gel electrophoresis assay (comet assay)

The comet assay was performed as described by Singh et al. with slight modifications (Singh et al., 1988). Briefly, a level, thin layer of agarose gel was prepared by dipping the slides in 1% warm agarose in PBS. 15 µl cell suspension ($1\text{-}2x10^4$ cells) were mixed with 85 µl of 0.5% low melting point agarose (LMPA) in PBS, placed on the first agarose gel layer, leveled with a cover glass, and allowed to solidify at 4°C for 5 min. The cover glass was removed and a third layer of 0.5% LMPA (100 µl) with a cover glass was applied. After the agarose solidified, the cover glass was removed and the glass slide was submerged in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO; pH 10) and kept for at least 60 min at 4°C to lyse the cells. The slide was transferred to an electrophoresis gel box filled with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH 13) for 20 min at room temperature to allow unwinding of DNA. Electrophoresis was conducted for 20 min at 25 V (0.8 V/cm) and 300 mA. Slides were washed in neutralisation buffer (0.4 M Trizma Base, pH 7.5) for 15 min and fixed in absolute ethanol for 5 min. The drained slides could be stored in the dark for several weeks. Before analysis the slide was stained with 50 µl ethidium bromide (20 µg/ml in distilled water) and covered with a cover glass.

Observations were made using a Zeiss fluorescence microscope (160x magnification) with a 515-560 nm excitation filter and a 590 nm barrier filter attached to a CCD camera. The extent of DNA migration was analysed using an image analysis system (Kinetic Imagining Ltd., U.K., Version 4.0).

2.4 Evaluation of DNA damage – statistics

The comet assay operates on the principle that DNA strand breaks create smaller DNA fragments that migrate more rapidly in an electric field than intact DNA. The migrating DNA forms a comet-shaped tail, which is morphologically distinct from the round head containing intact DNA. To express the results, the tail moment (TM), defined as the product of the percentage of DNA in the comet tail multiplied by the tail length (Olive et al., 1990), was used. The median TM of 50 randomly selected cells per experimental point (per slide) was determined. Each replicate experiment was performed on a different day with separately cultured cells. The mean TM (± standard deviation, SD) from 3-8 single experiments was calculated. Comparisons between non-exposed control group and H_2O_2 treated cells were performed by two-tailed Student's t-test for independent samples; values of $p<0.05$ and less were considered significant. Pair-wise comparison of each dose group against the concurrent control to identify significant effects at individual doses was recommended by Tice et al. (Tice et al., 2000).

2.5 Cell viability

Cell viability in non-exposed control and after treatment with hydrogen peroxide

was measured by the colorimetric microculture MTT-assay (Mossmann, 1983). In this assay, the reduction of the tetrazolium salt MTT ([3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide]) by mitochondrial succinate dehydrogenase to formazan crystals in viable, metabolically active cells is monitored. After treatment with H_2O_2, cells were allowed to incubate in 2 ml fresh culture medium for a period of 4 hours at 37°C and 5% CO_2. Trypsinised cells were resuspended in 600 µl medium. 50 µl of this cell suspension and 150 µl medium per well were seeded in triplicate into 96-well plates and incubated at 37°C in 5% CO_2 for 24 hours. The MTT-assay was performed essentially as described by Alley et al. (Alley et al., 1988). Briefly, after incubation, 50 µl MTT solution (Sigma; 0.5 mg/ml in PBS) was added to each well and cultures were incubated for a further 4 h. Then, medium was carefully aspirated and formazan crystals were dissolved in 150 µl dimethyl sulfoxide (DMSO; Merck) and 25 µl of Soerensen's glycine buffer (Plumb et al., 1989). After 10 min agitation on a shaker, the light absorbance was measured using a microtiter plate reader (Tecan, Germany) at wavelengths of 550 nm (test) and 620 nm (reference). Cell viability was expressed as a percentage of control absorbance after deduction of background.

3 Results

Significant DNA damage could be induced reproducibly in CAEC cells after exposure to 50 µM H_2O_2 for 10 min at 4°C, 20°C or 37°C (Tab. 1). There were no differences between TM measured after treatment at different temperatures. At concentrations of 100 µM H_2O_2 and above only cells with completely fragmented DNA without a visible nucleus were seen. Such cells, also called "clouds" or "hedgehog comets" with non-detectable cell nuclei could not be quantitatively evaluated by the analysis system.

Figure 1 shows representative histograms of tail moments for control (no hydrogen peroxide), and for 25 µM, 50 µM and 75 µM H_2O_2 treated cells. In the control group more than 95% of cells had TM values below 2.0. After treatment with 75 µM H_2O_2, all cells on the slides had visible comets. TM above 3.0 was measured in all these cells. The results show that CAEC respond homogenously to H_2O_2 exposure and damage was induced in the entire cell population.

Table 2 shows the effects of catalase on H_2O_2-induced DNA damage in CAEC. Significant protection is seen in the presence of catalase, the induction of comets after exposure to 25 µM, 50 µM or 75 µM H_2O_2 was abolished.

For the study of DNA repair, the time-dependent removal of lesions (i.e., the decrease in DNA migration after H_2O_2 exposure) was monitored over 15-240 min. The results are presented in Table 3. The time needed for DNA repair depended on the initial grade of damage. After 30 min post-incubation at 37°C, DNA damage was completely repaired in cells treated with 50 µM H_2O_2. Cell samples initially damaged with H_2O_2 concentrations between 100 µM and 400 µM were repaired after 60 min. After this time there were no more comets or cells with undetectable cell nuclei as seen immediately after exposure and the TM values measured in all groups were not significantly different from the control.

Cell viability after treatment with different H_2O_2 concentrations was measured using the MTT-assay. The results in Table 3 show that H_2O_2 exposure was not cytotoxic in this regard. Cell growth in samples exposed to 50 µM,

Tab. 1: DNA damage after treatment of human coronary artery endothelial cells with varying doses of hydrogen peroxide for 10 min at different temperatures.

Treatment	Tail moment		
with H_2O_2	4°C	20°C	37°C
control	0.37 ± 0.27 (4)	0.24 ± 0.18 (7)	0.46 ± 0.25 (6)
10 µM	0.32 ± 0.22 (4)	0.53 ± 0.46 (6)	0.81 ± 0.28 (3)
25 µM	1.04 ± 0.73 (4)	1.08 ± 0.53* (7)	0.96 ± 0.58 (3)
50 µM	7.50 ± 2.27* (3)	6.22 ± 2.08* (7)	5.76 ± 1.54* (4)
75 µM	6.35 ± 3.20* (4)	8.08 ± 2.57* (6)	6.50 ± 2.82* (3)

Mean values ± SD are presented, number of experiments is given in brackets.
* significantly greater than control value; $p<0.05$

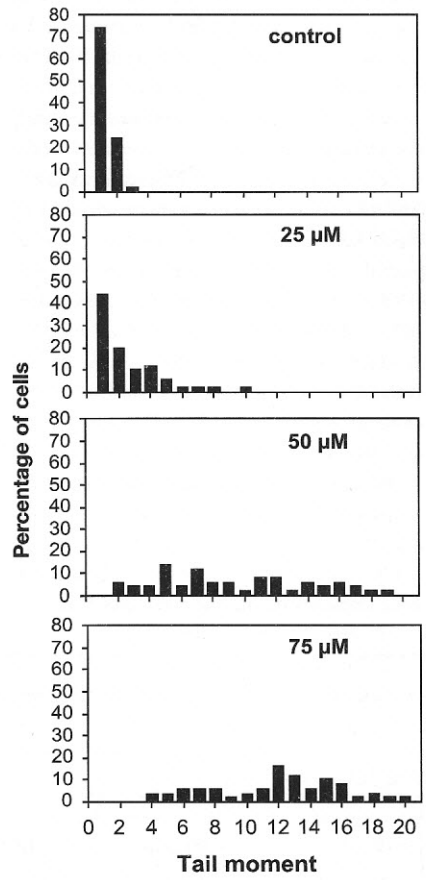

Fig. 1: Representative histograms of tail moment to show the distribution of DNA damage induced in human coronary artery endothelial cells after exposure for 10 min at 20°C to different concentrations of hydrogen peroxide. Data based on 50 cells/dose.

Tab. 2: Protective effect of catalase on H_2O_2-induced oxidative damage in human coronary artery endothelial cells.

		Tail moment*	
H_2O_2 concentration	Experiment	without catalase	with catalase
control	1	0.57	0.38
	2	0.38	0.29
25 µM	1	1.66	0.2
50 µM	1	3.60	0.19
	2	7.48	0.35
75 µM	1	5.59	0.42

Cells were treated with H_2O_2 in presence or absence of catalase for 10 min at 20°C.
* Median values from 50 cells per experimental point.

200 µM or 400 µM H_2O_2 was not different to non-exposed control cells.

4 Discussion

Oxidative DNA damage and consequent dysfunction in endothelial cells was first studied by Spragg (Spragg, 1991) in bovine pulmonary arteries and aortas. Ballinger et al. (2000) studied oxidative mitochondrial DNA damage in endothelial cells from human umbilical veins. On the basis of their experiments the authors assumed that such ROS-induced mitochondrial damage could possibly mediate endothelial and vascular dysfunction and so promote atherogenesis development.

Previously, we established a cell culture model for systematic investigation of oxidative DNA damage and repair in human coronary smooth muscle cells (Kreja and Finking, 2002). With the present study an *in vitro* model for such investigations in human coronary endothelial cells (CAEC) was to be established.

Measurable DNA damage in CAEC could be induced after exposure to 50 µM H_2O_2 for 10 min. These results are comparable to those presented by Spragg (1991) who had quantified substantial DNA strand breakage in bovine pulmonary artery and aortic endothelial cells by a fluorometric method after treatment with 50 µM H_2O_2. According to Spragg, this concentration can be found at the interface between the adherent leukocyte and the vascular endothelial cell.

The results of our present study show dose-dependent damage of DNA in endothelial cells (Fig.1, Tab.1). At concentrations of 100 µM H_2O_2 and above DNA was completely fragmented. However, even after exposure to high concentrations of H_2O_2 endothelial cells had a high capacity to repair oxidative DNA damage. After 60 min even the highest grades of damage induced with doses up to 400 µM H_2O_2 were repaired (Tab. 3). This is important for *in vivo* situations as well because during normal cell metabolism the amount of oxidative damage is estimated at tens of thousands of hits per cell per day (Ames and Gold, 1990). However, the measurement of the damage repair rates, as performed in the present study, did not provide any information about the repair fidelity. DNA strand break misrepair could not be excluded. If damage in crucial regions of DNA is not repaired or not repaired properly, malignant cell transformation or mutations can occur as a consequence of such repair defects. The involvement of somatic mutations in the formation of atherosclerotic plaques was first suggested by Benditt and Benditt (1973). They proposed the "monoclonal" hypothesis of atherosclerosis and postulated that a plaque may be regarded as a monoclonal benign neoplasm of the artery wall. Studies on DNA damage and repair mechanisms in atherosclerotic tissue are lacking. However, there is accumulating evidence that somatic mutations play a role in the development of atherosclerotic plaques. The study of DNA alterations may be of fundamental impor-

Tab. 3: Repair kinetics of induced DNA damage and cell viability after exposure of human coronary artery endothelial cells to hydrogen peroxide.

	Tail moment after H_2O_2 treatment						
Treatment with H_2O_2	0 min	15 min	30 min	60 min	120 min	240 min	cell viability*
control	0.15 ± 0.01 (15)	0.15 ± 0.05 (2)	0.13 ± 0.04 (2)	0.13 ± 0.76 (4)	0.15 ± 0.05 (9)	0.12 ± 0.01 (2)	100
50 µM	6.56 ± 2.71** (8)	2.43 ± 1.27** (3)	0.62 ± 0.50 (8)	0.33 ± 0.19 (9)	0.21 ± 0.06 (5)		90 ± 12
100 µM	NDCN	3.47	4.32 ± 3.16** (4)	0.48 ± 0.32 (4)	0.20 ± 0.11 (2)		78 ± 3**
200 µM	NDCN	10.24	1.27 ± 1.59** (4)	0.41 ± 0.15 (4)	0.32 ± 0.21 (2)		84 ± 14
300 µM	NDCN			0.47 ± 0.37 (4)	0.21 ± 0.11 (4)	0.16 ± 0.06 (2)	
400 µM	NDCN			0.47 ± 0.31 (4)	0.21 ± 0.10 (4)	0.15 ± 0.04 (2)	101 ± 12

Cells were treated with H_2O_2 for 10 min at 20°C and than incubated in fresh medium for 15-240 min at 37°C.
Mean values (± SD) are presented; number of experiments is given in brackets.
*Percentage of viable cells vs. non-exposed control, as measured in MTT assay; mean values from 3 experiments ± SD.
**significantly different to the control value; $p<0.05$ and less
NDCN = non-detectable cell nuclei; cells with completely fragmented DNA

tance for understanding the mechanisms of the development of atherosclerosis (Andreassi, 2003; Andreassi et al., 2000; Bridges et al., 1990; Penn, 1990).

Oxidative damage induced by H_2O_2 could be prevented by additional treatment with catalase (Tab. 2). This demonstrates the importance of antioxidant protection of endothelial cells and other cells in the vascular wall. Antioxidant protection against free radical damage by means of enzymes like catalase or peroxidases is permanently active *in vivo*. It may be supported by nutritional components. Estrogens and phytoestrogens are considered to play a protective role in cells of the cardiovascular system (Finking et al., 2001a; Finking et al., 1999a; van der Schouw et al., 2000; Wisemann, 2000). One well investigated target of such antioxidative protection is oxidised LDL, which is a key promoter of atherosclerosis (Hwang et al., 2000). Usually such mechanisms and effects on endothelial cell function are investigated not only *in vitro* but also in animal models (Chandrasekar et al., 2001; Finking et al., 2001b; Holm et al., 1998).

The cell culture model introduced here offers a variety of possibilities to work systematically on questions of potentially ROS-induced and mutagenic aspects of human coronary atherosclerosis. Steroid hormones like 17ß-estradiol (Finking et al., 2001a) or phytoestrogens (Finking et al., 1999a) which have antioxidant properties can be investigated as modulators in this regard. It has frequently been demonstrated that such agents act antiatherogenically and antiproliferatively in mouse (Sullivan et al., 1995), rat (Oparil et al., 1997), rabbit (Hanke et al., 1996; Finking and Hanke, 1997; Finking et al., 2001b), swine (Shi et al., 1996) and primate animal models (Holm et al., 1998) as well as in organ culture experiments (Vargas et al., 1993; Finking et al., 1999b; Finking et al., 2000). Whether antioxidative effects are involved in this action requires more investigation on the cellular level. Other potent antioxidative agents to be investigated are vitamin C and E or artificial antioxidants like probucol (Bhavnani et al., 2001). However, most studies in this regard focus on alterations of oxidised low density lipoprotein (oxLDL), macrophages and atherosclerotic plaques but not on mutagenic, DNA-altering processes. Animal, i.e., rabbit models, because of their complexity, come to rather controversial results regarding antioxidative vitamin actions and their physiological effects (Upston et al., 2002; Yoshida et al., 2002).

With the cell culture model presented we can provide an *in vitro* system to investigate distinct mechanisms, which are – to date – poorly analysed and understood. By using human coronary endothelial cells the experiments can target the relevant cells directly. Animals are not required as direct objects of such experiments or donors of tissue and cells.

References

Alley, M. C., Scudiero, D. A., Monks, A. et al. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res. 48*, 589-601.

Ames, N. N. and Gold, L. S. (1990). Chemical carcinogenesis: too many carcinogens. *Proc. Natl. Acad. Sci. USA 87*, 7772-7776.

Andreassi, M. G. (2003). Coronary atherosclerosis and somatic mutations: an overview of the contributive factors for oxidative DNA damage. *Rev. Mutat. Res. 543*, 67-86.

Andreassi, M. G., Botto, N. and Colombo, M. G. (2000). Genetic instability and atherosclerosis: can somatic mutations account for the development of cardiovascular diseases? *Environ. Mol. Mutagen. 35*, 265-269.

Baker, M. A. and He, S. Q. (1991). Elaboration of cellular DNA breaks by hydroperoxides. *Free Radic. Biol. Med. 11*, 563-572.

Ballinger, S. W., Patterson, C., Yan, C. N. et al. (2000). Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ. Res. 86*, 960-966.

Benditt, E. and Benditt, J. (1973). Evidence for monoclonal origin of human atherosclerotic plaques. *Proc. Natl. Acad. Sci. (USA) 70*, 1753-1756.

Bhavnani, B., Cecutti, A. and Gerulath, A. (2001). Comparison of the antioxidant effects of equine estrogens, red wine components, vitamin E, and probucol on low-density lipoprotein oxidation in postmenopausal women. *Menopause 8*, 408-419.

Bridges, B. A., Bowyer, D. E., Hansen, E. S. et al. (1990). The possible involvement of somatic mutations in the development of atherosclerotic plaques. *Mutat. Res. 239*, 143-148.

Chandrasekar, B., Nattel, S. and Tanguay, J. F. (2001). Coronary artery endothelial protection after local delivery of 17beta-estradiol during balloon angioplasty in a porcine model: a potential new pharmacologic approach to improve endothelial function. *J. Am. Coll. Cardiol. 38*, 1570-1576.

Collins, A., Dobson, V., Dusinska, M. et al. (1997). The comet assay: what can it really tell us? *Mutat. Res. 375*, 183-193.

Collins, A. R. (2002). The Comet assay. Principles, applications, and limitations. *Meth. Mol. Biol. 203*, 163-177.

Collins, A. R., Duthie S. J. and Dobson, V. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis 14*, 1733-1735.

Finking, G., Gohar, M. H., Lenz, C. and Hanke, H. (2001a). Cardiovascular oestrogen effects in women. *J. Obstet. Gynecol. 21*, 340-349.

Finking, G. and Hanke, H. (1997). Nikolaj Nikolajewitsch Anitschkow (1885-1964) established the cholesterol-fed rabbit as a model for atherosclerosis research. *Artherosclerosis 135*, 1-7.

Finking, G., Hess, B. and Hanke, H. (1999a). The value of phytoestrogens as a possible therapeutic option in postmenopausal women with coronary heart disease. *J. Obst. Gynecol. 19*, 455-459.

Finking, G., Krauss, N. and Römer, S. (2001b). 17beta-estradiol, gender independently, reduces atheroma development but not neointimal proliferation after balloon injury in the rabbit aorta. *Atherosclerosis 154*, 39-49.

Finking, G., Wohlfrom, M. and Lenz, C. (1999b). The phytoestrogens Genistein und Daizein and 17 beta-estradiol inhibit development of neointima in aortas from male and female rabbits in vitro after injury. *Coron. Artery Dis. 10*, 607-615.

Finking, G., Wolkenhauer, M., Lenz, C. and Hanke, H. (2000). Post-injury ex vivo model to investigate effects and toxicity of pharmacological treatment in rings of rabbit aortic vessels. *ALTEX 17*, 67-74.

Fuster, V., Badimon, L. and Badimon, J. J. (1992a). The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *N. Engl. J. Med. 326(4)*, 242-250.

Fuster, V., Badimon, L. and Badimon, J. J. (1992b). The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N. Engl. J. Med. 326(5)*, 310-318.

Hanke, H., Hanke, S. and Finking, G. (1996). Different effects of estrogen and progesterone on experimental atherosclerosis in female versus male rabbits. Quantification of cellular proliferation by bromodeoxyuridine. *Circulation 94*, 175-181.

Holm, P., Andersen, H. L., Arre, G. and Stender, S. (1998). Gender gap in aortic cholesterol accumulation in cholesterol-clamped rabbits: role of the endothelium and mononuclear-endothelial cell interaction. *Circulation 98*, 2731-2737.

Hwang, J., Sevanian, A., Hodis, H. and Ursini, F. (2000). Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid. *Free Radic. Biol. Med. 29*, 79-89.

Krasinski, K., Spyridopoulos, I., Asahara, T. et al. (1997). Estradiol accelerate functional endothelial recovery after arterial injury. *Circulation 95*, 1768-1172.

Kreja, L. and Finking, G. (2002). Development of an in vitro model to study oxidative DNA damage in human coronary smooth muscle cells. *ALTEX 19*, 123-129.

Ludden, J. B., Bruger, M. and Wright, I. S. (1942). Effect of testosterone propionate and estradiol dipropionate on experimental atherosclerosis in rabbits. *Arch. Pathol. 33*, 58-62.

Merk, O. and Speit, G. (1999). Detection of cross links with the comet assay in relationship to genotoxicity and cytotoxicity. *Environ. and Molecular Mutagenesis 33*, 167-172.

Mossmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth. 65*, 55-63.

Olive, P. L., Banáth, J. P. and Durand, R. E. (1990). Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet assay". *Radiat. Res. 122*, 86-94.

Oparil, S., Levine, R. and Chen, S. (1997). Sexually dimorphic response of the balloon-injured rat corotid artery to hormone treatment. *Circulation 95*, 1301-1307.

Penn, A. (1990). Mutational events in the etiology of arteriosclerotic plaques. *Mutat. Res. 239*, 149-162.

Plumb, J., Milroy, R. and Kaye, S. (1989). Effects of pH dependence of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res. 49*, 4435-4440.

Rojas, E., Lopez, M. C. and Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *J. Chromatogr. B. Biomed. Sci. Appl. 722*, 225-54.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature 362*, 801-809.

Ross, R. (1999). Atherosclerosis – an inflammatory disease. *N. Engl. J. Med. 340*, 115-126.

Shi, Y., O'Brien, J. and Fard, A. (1996). Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. *Circulation 94*, 1655-1664.

Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell. Res. 175*, 184-91.

Spragg, R. G. (1991). DNA strand break formation following exposure of bovine pulmonary artery and aortic endothelial cells to reactive oxygen products. *Am. J. Resp. Cell. Mol. Biol. 4*, 4-10.

Sullivan, T., Karas, R. and Aronowitz, M. (1995). Estrogen inhibits the response to injury in a mouse caroid artery model. *J. Clin. Invest. 96*, 2482-2488.

Sundaresan, M., Yu, Z. X., Fererans, V. J. et al. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science 270*, 296-299.

Tice, R. R., Agurell, E., Andersson, B. et al. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutag. 35*, 206-221.

Upston, J., Terentis, A. and Morris, K. (2002). Oxidized lipid accumulates in the presence of alpha-tocopherol in atherosclerosis. *Biochem. J. 363*, 753-760.

van der Schouw, Y. T., de Kleijn, M. J., Peeters, P. and Grobbee, D. E. (2000). Phyto-oestrogens and cardiovascular disease risk. *Nutr. Metab. Cardiovasc. Dis. 10*, 154-167.

Vargas, R., Wroblewska, B. and Rego, A. (1993). Oestradiol inhibits smooth muscle cell proliferation of pig coronary artery. *Br. J. Pharmacol. 109*, 612-617.

Wisemann, H. (2000). The therapeutic potential of phytoestrogens. *Exp. Opin. Invest. Drugs. 9*, 1829-1840.

Yoshida, N., Murase, H. and Kunieda, T. (2002). Inhibitory effect of a novel water-soluble vitamin E derivate on atherosclerosis in rabbits. *Atherosclerosis 162*, 111-117.

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